

The Degradation of Hyaluronic Acid by Ascorbic Acid

By DAVID A. SWANN

*Department of Connective Tissue Research, Institute of Biological and Medical Sciences,
Retina Foundation, Boston, Mass., U.S.A.*

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Hyaluronic acid, a constituent of the ground substance of connective tissues, is known to be depolymerized by ascorbic acid. The literature has been reviewed by Sundblad & Balazs (1966). Heavy-metal ions such as those of copper and iron and also hydrogen peroxide have often been included as either catalysts or components in the reaction, and, because other carbohydrates are also degraded by these systems, it has been suggested by several workers that the mechanism by which the reaction occurs involves the production of hydroxyl radicals, which are known to attack carbohydrate structures (Phillips, 1961). In the study of this reaction now reported, the depolymerization of hyaluronic acid by ascorbic acid has been performed in the absence of added substances and at pH 3.0, under which condition ascorbic acid is relatively stable.

The hyaluronic acid used was prepared from rooster combs (fraction 2; D. A. Swann, unpublished work) and was purified by centrifugation and repeated ethanol precipitation after treatment of hyaluronic acid solutions with chloroform. A final treatment with saturated NaCl was used to 'salt-out' extraneous proteins (Laurent, 1955). The final product had a molecular weight of 1.25×10^6 , calculated from the sedimentation and diffusion coefficients after extrapolation to zero concentration, an intrinsic viscosity in 0.2M-NaCl of 3000 ml./g. and a protein content, by determination of the amino acid content, of 3.0% of the complex. The glucuronic acid content of the hyaluronic acid was determined by an automated carbazole reaction in the presence of borate (Dische, 1947; Gregory, 1960; Balazs, Berntsen, Karossa & Swann, 1965a) and the glucosamine content by an automated *p*-dimethylaminobenzaldehyde reaction (Elson & Morgan, 1933; Balazs, Berntsen, Karossa & Swann, 1965b). The hyaluronic acid concentration of solutions was calculated from the glucuronic acid value $\times 1.95$.

Before use, the hyaluronic acid was dialysed extensively against deionized-distilled water and then against a suspension of Dowex 50 (X8) ion-exchange resin in the H⁺ form that had been carefully washed so that the washings had the same pH as the deionized water. The pH of the hyaluronic acid solutions after this treatment varied between

2.9 and 3.1. Freshly prepared solutions of L-ascorbic acid (Baker Analyzed Reagent; J. T. Baker Chemical Co., Phillipsburgh, N.J., U.S.A.) were added to constant amounts of hyaluronic acid to give hyaluronic acid/ascorbic acid ratios (mg./mg.) between 1 and 40 in the same volume of the mixture, with a hyaluronic acid concentration of 1 mg./ml.

The quantity of ascorbic acid in the hyaluronic acid-ascorbic acid mixtures can be followed spectrophotometrically because the enediol group of ascorbic acid at pH 3.0 absorbs in the ultraviolet with an absorption maximum at 243 m μ . An initial experiment with a hyaluronic acid/ascorbic acid ratio 10:1 showed a direct relationship between the decrease in E_{243} and the viscosity decrease with time, which implies that the viscosity decrease is dependent on the ascorbic acid concentration. This was confirmed in later experiments (Table 1), where the decrease in relative viscosity after 5 min. was dependent on the quantity of ascorbic acid present in the mixture.

When infrared spectra of thin films of ascorbic acid-degraded hyaluronic acid were measured (Perkin Elmer 237 spectrophotometer), a new absorption maximum was observed at a frequency of 1800 cm.⁻¹. Ascorbic acid does not have an

Table 1. *Dependence of the viscosity decrease and the appearance of the new absorption maximum at 1800 cm.⁻¹ in the infrared spectrum of hyaluronic acid-ascorbic acid mixtures on the concentration of ascorbic acid*

Hyaluronic acid/ ascorbic acid ratio (mg./mg.)	Relative viscosity after 5 min. (% of the original)	Extinction at 1800 cm. ⁻¹ (% of extinction at 1735 cm. ⁻¹)
1:1	25	—
2:0	—	73
2:6	—	50
3:6	—	37
6:0	—	23
10:6	46	15
21:2	52	—
25:0	60	—
30:0	66	—
40:0	72	—

absorption maximum at this frequency, but dehydroascorbic acid does. It is thought therefore that the occurrence of the new absorption maximum at 1800 cm^{-1} was caused by the production of dehydroascorbic acid from ascorbic acid during the degradation reaction. This assumption is supported by the fact that the extinction at 1800 cm^{-1} compared with that at 1735 cm^{-1} , which is the main absorption peak for the CO_2H group in hyaluronic acid (Orr, 1954), is proportional to the quantities of ascorbic acid added to the hyaluronic acid. The 1800 cm^{-1} absorption maximum also disappears after dialysis of the ascorbic acid-degraded hyaluronic acid.

In an early report by Pigman & Rizvi (1959), dehydroascorbic acid was stated to be inactive as a hyaluronic acid-degrading agent. More recently it was reported that dehydroascorbic acid was nearly as effective as ascorbic acid in its ability to degrade hyaluronic acid (Matsumura, Herp & Pigman, 1966). Under the conditions of the experiments now reported, dehydroascorbic acid (K & K Laboratories, Plainview, N.Y., U.S.A.) was virtually inactive; there was only a 4% decrease in relative viscosity in 40 min. compared with an 83% decrease for ascorbic acid when hyaluronic acid/ascorbic acid and hyaluronic acid/dehydroascorbic acid ratios 1.3:1 were used.

Confirming an earlier report (Pigman & Rizvi, 1959), no diffusible products were produced in the reaction and there was a quantitative recovery of glucuronic acid and glucosamine both after dialysis and after chromatography on ECTEOLA-cellulose. The degraded hyaluronic acid had only a slightly lower mobility in free electrophoresis ($7.9 \times 10^{-5} \text{ cm}^2 \text{ sec}^{-1} \text{ v}^{-1}$ compared with $8.9 \times 10^{-5} \text{ cm}^2 \text{ sec}^{-1} \text{ v}^{-1}$ for the original hyaluronic acid) and it had the same optical rotation ($[\alpha]_D^{20} -74^\circ$) and infrared spectrum as the starting material.

The amino acid content of the hyaluronic acid, however, was decreased markedly after treatment with ascorbic acid. Before degradation a total of 90 m-moles of amino acids/mole of glucuronic acid was present in the complex, and the acid and neutral amino acids present in the largest amounts were aspartic acid (8.5 m-moles), serine (8.1 m-moles), glutamic acid (10.5 m-moles), glycine (10.0 m-moles) and alanine (7.7 m-moles/mole of glucuronic acid). On treatment with ascorbic acid, a precipitate was formed that could be collected by centrifugation and that contained about 70% of the amino acids originally present in the hyaluronic acid. Analysis of the soluble hyaluronic acid in the supernatant fraction showed that most of the acid and neutral amino acids were decreased to 20% of their original concentration (expressed as m-moles/mole of glucuronic acid), whereas three amino acids, namely serine, glycine and alanine, were still

present in quantities of more than 40% of the original hyaluronic acid. The concentrations of serine, glycine and alanine in one sample of ascorbic-acid-degraded hyaluronic acid were 3.59, 4.67 and 3.12 m-moles/mole of glucuronic acid respectively.

These experiments support the view that rooster-comb hyaluronic acid is degraded by a simple oxidation-reduction mechanism involving the conversion of ascorbic acid into dehydroascorbic acid. Because no diffusible glucuronic acid or glucosamine constituents were observed and the intrinsic viscosity of the ascorbic acid-degraded hyaluronic acid reached a limit with a minimum of 90–100 ml./g., even when excess of ascorbic acid was present, it is suggested that certain linkages in the hyaluronic acid macromolecule are sensitive to reduction by ascorbic acid. If the degradation reaction were a random process in which the carbohydrate chain of hyaluronic acid was depolymerized by the cleavage of glycosidic linkages, then small diffusible substances would almost certainly be formed. Such a mechanism would, however, not account for the incomplete degradation of the carbohydrate chains of hyaluronic acid in the presence of excess of ascorbic acid. If the depolymerization occurred by a destruction of the constituent sugars in the hyaluronic acid, then hyaluronic acid must contain sugar units other than glucuronic acid and glucosamine because there was no apparent destruction of these constituents when colorimetric methods of analysis were used.

An alternative hypothesis is suggested by the drastic decrease in the amino acid content of the hyaluronic acid after treatment with ascorbic acid. Although part of the protein material precipitated from the hyaluronic acid solutions after treatment with ascorbic acid may have been trapped in the hyaluronic acid macromolecule and merely been released after degradation, the different amino acid composition of the ascorbic acid-degraded hyaluronic acid and the significant intrinsic viscosity (90–100 ml./g.) of this material indicate that the site of the reaction may be located in a protein moiety of the macromolecule.

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